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(54) Title: POLYMERIC CARRIERS FOR RELEASE OF COVALENTLY LINKED AGENTS

(57) Abstract

A chemically defined polymeric carrier comprising a series of α-amino acids in any combination containing side chains to which diagnostic/therapeutic and chelating agents can be covalently joined through cleavable linkers either directly or covalently joined through cleavable linkers after chemical modification of the side chains. Hydrazone, disulfide, and ester linkages in any combination can be present in the polymeric carrier between the side chains of the α-amino acids and the agents. The presence of a particular covalent linkage between the side chain and the agent in the carrier is determined by the functional group present in the side chain of the α-amino acid and the functional group present in the agent. The α-amino acids with side chains to which agents do not covalently join can function as spacers to minimize interaction between bulky molecules attached to the polymeric carrier. In addition, those α-amino acids with charge or hydrophilic side chains to which agents do not covalently join can provide increased solubility to the polymeric carrier.

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POLYMERIC CARRIERS FOR RELEASE OF COVALENTLY LINKED AGENTS

BACKGROUND OF THE INVENTION

1. Field of the Invention

5   The invention relates to chemically defined polymeric
carriers that provide advantageous properties for _in vivo_
imaging and therapy. The polymeric carriers consist of
α-amino acids that contain side chains covalently joined
to (i) diagnostic and therapeutic molecules and (ii)

10   chelating agents capable of binding diagnostic or
therapeutic radionuclides.

2. Related art

Monoclonal antibodies have been developed that localize in

15   cancerous tissue, due to their high specificity and
affinity for antigens on tumor cell surfaces.   This
development has increased the prospect of clinical
applications, if such antibodies can be linked to
diagnostic and therapeutic agents. The high specificity

20   of the antibodies makes them desirable candidates as
targeting molecules for delivering a diagnostic or
therapeutic agent to a cancer site.

Unfortunately, the direct linkage of such agents to an

25   antibody weakens its immunoreactivity.   Any deriv-
atization of the antibody weakens its immunoreactivity.
Therefore, an antibody with multiple linkages to
diagnostic or therapeutic agents is an antibody with low
specificity. At the present time, chelating agents, which

30   bind to diagnostic and therapeutic radionuclides, are

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dir ctly linked to antibodies. This precludes th
controlled release of the radionuclide from the
antibodies. Due to the large size of the antibody
molecule, it is difficult, using conventional detection
5   techniques, to determine the exact nature of the chelating
agent linkages to the antibody, and to determine the
number of chelating agents linked to the antibody. A lack
of such precise information presents problems for getting
regulatory approval of chelating agents. Regulatory
10   agencies require that all substances subject to their
approval must include information that clearly identifies
the structure of the substance, which is to be introduced
into the body.

15   What is needed is an approach that derivatizes a targeting
molecule, such as an antibody, at a minimum number of
sites to carry larger amounts of diagnostic and
therapeutic agents. Also needed, is an approach that can
determine the nature of the chelating agent linkage to the
20   antibody and the number of chelating agents linked to the
antibody.

SUMMARY OF THE INVENTION

The present invention provides a chemically defined polymeric carrier. It comprises a series of α-amino acids, in any combination, containing side chains to which diagnostic/therapeutic and chelating agents can be covalently joined through cleavable linkers. The agents can be covalently joined to the side chains through cleavable linkers either directly or covalently joined to the side chains through cleavable linkers after chemical modification of the side chains. Hydrazone, disulfide, and ester linkages, in any combination, can be present in the polymeric carrier between the side chains of the α-amino acids and the agents. The selection of a particular covalent linkage between the side chain and the agent in the polymeric carrier is determined by the functional group in the α-amino acid side chain and the reactive functional group in the agent. The α-amino acids with side chains to which agents do not covalently join can function as spacers, to minimize interaction between the bulky molecules attached to the polymeric carrier. In addition, those α-amino acids with charged or hydrophilic side chains to which agents do not covalently join can provide increased solubility to the polymeric carrier.

N-terminal protecting groups which are optional for the polymeric carrier include all the standard amine protecting groups. C-terminal conjugation groups which are optional for attachment of the polymeric carrier to the targeting molecule include all conjugation groups known in the art. In order to provide efficient attachment f the polymeric targeting molecule, a spacer

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group is present in the polymeric carrier between the α-amino acids and the conjugation group. The spacer group presents any steric hindrance to the attachment by any agent appended from the C-terminal end of the carrier.

5   These spacer groups are terminal aminoacids, such as γ-aminobutyric acid (Aba). In the absence of the conjugation group, the spacer group, e.g., Aba through its carboxyl group, can attach the polymeric carrier to the targeting molecule.

10

The peptides that constitute the polymeric carrier are prepared from α-amino acids by conventional solution methods or by solid-phase peptide synthesis. These peptides have been modified to carry derivatized

15   diagnostic/ therapeutic agents, and chelating agents that bind to diagnostic and therapeutic radionuclides. These agents can be released either at the target site or after internalization by the cell.

20   Many advantages arise from the present invention. The polymeric carrier can carry a maximum number of agents while derivatizing a targeting molecule at a minimum number of sites. Thus the biological activity of the targeting molecule is maintained at a high level, even

25   though it is attached to multiple agents. For example, the fewer the linkages in an antibody, the higher is its specificity.

The rate at which agents can be released from the

30   polymeric carrier attached to the targeting molecule is controlled by manipulating the nature of the covalent linkages in the polymeric carrier. For example, by adjusting the stability of the covalent linkages or by

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using different types of covalent linkages on a polymeric carrier, agents--arc released at a mixed rate. This is particularly important when the disease requires use of long term diagnostics or therapeutics.

Multiple agents, which may be the same or different, are attached to the polymeric carrier. Not only can the same agents be released at a mixed rate, but different agents can be released at a mixed rate in the same target site.

The polymeric carrier, with its covalently linked agents, is a relatively small molecule compared to the targeting molecule. Therefore, conventional detection techniques can predetermine the exact nature of the agent linkages to the targeting molecule before the polymeric carrier is attached to it. In addition, radiolabeling techniques can determine the precise number of polymeric carriers linked to the targeting molecule.

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BRIEF DESCRIPTI N OF THE FIGURES

Figure 1 is a flow chart representing the general procedure for synthesis of a polymeric carrier with attached agents and the conjugation of the polymeric carrier to an antibody.

Figure 2 is a flow chart representing a procedure for the synthesis of a polymeric carrier with attached therapeutic agents and the conjugation of the polymeric carrier to an antibody.

Figure 3 illustrates the removal of protecting groups from amino acid side chains of a polymeric carrier.

Figures 4 and 6 are flow charts representing procedures for the synthesis of polymeric carriers with attached chelating agents and the conjugation of the polymeric carriers to antibodies.

Figure 5 is a flow chart representing a procedure for the synthesis of a chelating agent.

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DETAILED DESCRIPTION OF THE INVENTI N

The present invention relates to a chemically defined polymeric carrier that increases the loading of diagnostic/therapeutic and chelating agents to targeting molecules. The polymeric carrier comprises a series of from 2 to about 18 α-amino acids in any combination that include side chains which can covalently join through cleavable linkers to agents. The number of agents covalently joined through cleavable linkers to the polymeric carrier can be from 2 to about 18. This number is determined by the number of α-amino acid side chains in the polymeric carrier available for covalent bonding through cleavable linkers to the agents.

The term "polymeric carrier" as used in the invention denotes a peptide carrier. Those α-amino acids whose side chains are not covalently joined to agents can function as spacers for the polymeric carrier. These spacers reduce any non-bonded interactions between agents attached to modified α-amino acids. In addition to acting as spacers, those α-amino acids with charged or hydrophilic side chains not covalently joined to agents can impart increased solubility to the polymeric carrier.

The polymeric carrier includes optionally a protecting group at its N-terminal end and optionally a conjugation group at its C-terminal end. The conjugation group enables the polymeric carrier to attach itself to a targeting molecule. A spacer group is placed between the α-amino acids and the conjugation group to aid in the attachment of the polymeric carrier to the targeting molecule. The spacer group prevents any steric hindranc to th

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attachment by any agent appended from the C-terminal end of the carrier. In addition, the spacer group, a terminal amino acid, may attach the polymeric carrier to the targeting molecule without the presence of the conjugation group. This may occur by reacting the carboxyl group of the terminal amino acid with functional groups on the targeting molecule to form covalent bonds, such as ester and amide linkages.
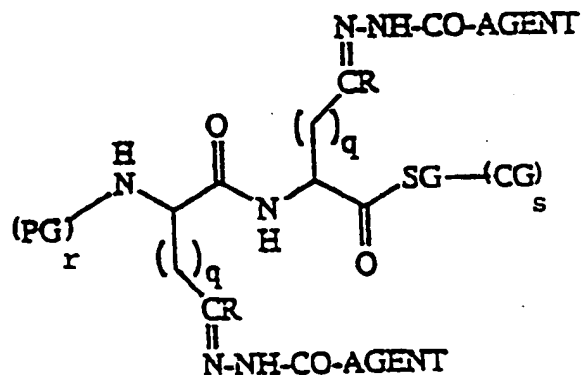
In the polymeric carrier, α-amino acids having side chains that enhance polarity and therefore, water solubility, are desirable. The increased water solubility is believed to further contribute to decreased hepatobiliary uptake of radiolabeled polymeric carrier proteins. The α-amino acids having side chains that enhance water solubility include those with charged side chains (lysine, arginine, histidine, cysteine, aspartic acid, glutamic acid, tyrosine, tyrosine-O-SO$_3$-) and those with hydrophilic side chains (serine, threonine, asparagine, glutamine). Standard amine protecting groups can be used for the N-terminal protecting group of the polymeric carrier. Preferred embodiments of the invention comprise acetyl, proprionyl, phenylacylsulfonyl, substituted phenyl-acylsulfonyl, and other hydrophilic protecting groups.

A conjugation group is a chemically reactive functional group that will react with a targeting molecule to bind the polymeric carrier thereto. When the targeting molecule is a protein, the conjugation group is reactive under conditions that do not denature or otherwise adversely affect the protein. Therefore, the conjugation group is sufficiently reactive with a functional group on

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a protein so that th reaction can be conducted in a substantially aqueous solution and does not have to be forced, e.g., by heating to high temperatures, which may denature the protein. Examples of suitable conjugation groups include but are not limited to active esters, isothiocyanates, amines, hydrazines, maleimides or other Michael-type acceptors, thiols, and activated halides. Among the preferred active esters are N-hydroxysuccinimidyl ester, sulfosuccinimidyl ester, thiophenyl ester, 2,3,5,6-tetrafluorophenyl ester, and 2,3,5,6-tetrafluorothiophenyl ester. The latter three preferred active esters may comprise a group that enhances water solubility, at the para (i.e., 4) or the ortho position on the phenyl ring. Examples of such groups are CO_2H, SO_3-, PO_3^{2-}, OPO_3^{2-}, OSO_3^-, N^+R_3 wherein each R represents H or an alkyl group, and $O(CH_2CH_2O)_nCH_3$ groups.

Terminal amino acids used as spacer groups in the invention include aminocaproic acid, aminopentanoic acid, γ-aminobutyric acid, β-alanine, glycine, and the like.

Agents containing hydrazides, $R(CO)NHNH_2$, react with α-amino acid side chains containing aldehydes, RCHO, or ketones, $R_2(CO)$, to form polymeric carriers with hydrazone linkages having the following formula:

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wherein   the α-amino acids in the polymeric carrier are
            from 2 to about 18 units;
            PG is an N-terminal protecting group;
            SG is a spacer group that by preventing steric
            hindrance by agents appended from the C-
            terminal end of the carrier promotes efficient
            attachment of the polymeric carrier to a
            targeting molecule;
            CG is a conjugation group useful for the
            attachment of the polymeric carrier to a
            targeting molecule;
            AGENT is from 2 to about 18 units of a
            diagnostic or therapeutic agent, or a chelating
            agent capable of binding diagnostic or
            therapeutic radionuclides in the polymeric
            carrier;
            R is H, CH_3, phenyl, or phenyl substituted with
            electron-donating and/or electron-withdrawing
            groups;
            q is 0 or 1;
            r is 0 or 1; and
            s is 0 or 1.

Hydrazone formation is an effective method of attaching
certain therapeutic agents to monoclonal antibodies (King
et al., Biochemistry, Vol. 25:5774, 1986). Recent work in
the area of therapeutic immunoconjugates addressed the
hydrazone functionality as a potentially cleavable linker
between a chemotherapeutic agent and a monoclonal
antibody. Laguzza et al., (J. Med. Chem., Vol. 32:548,
1989) demonstrated that a vinca alkaloid can be conjugated
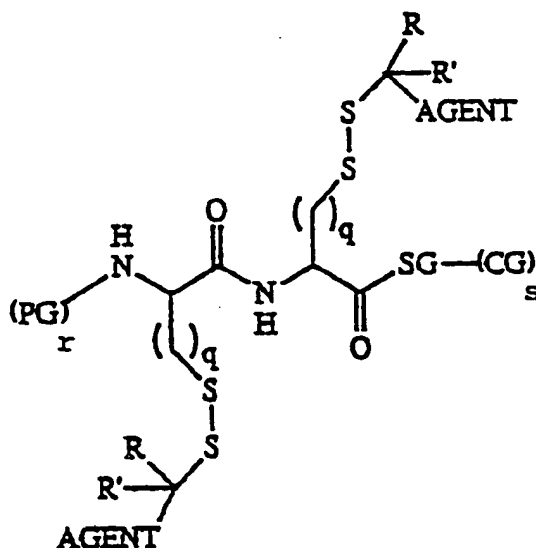to an antibody via a hydrazone linkage and that pH

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dependency of the drug could be studied. The hydrazone linkage approach was based on the premise that a conjugate formed via a serum stable, yet acid-labile hydrazone linker would serve the purpose of delivering the drug conjugate to the tumor site and then slowly release upon exposure to the tumor's acidic environment (Tannock et al., Cancer Research, Vol. 49:4373, 1989). This conditional requirement necessitated the screening of several small molecule hydrazones to evaluate their stability in human serum and acetate buffer at pH 5.6.

The design of the polymeric carrier system with a hydrazone linkage incorporates the observed results of a small molecule study. Peptides of known amino acid sequences are constructed to carry primary or secondary hydroxyl groups (the chain may carry more than one hydroxy amino acid (primary or secondary), which can be oxidized to the carbonyl compound.

The results of the small molecule study indicate that hydrazones from aromatic aldehydes may be too stable to be useful. Hydrazones derived from aliphatic ketones have a serum half-life of 15-20 hours (generated in the peptides from threonine and other aminoacids containing secondary -OH groups). Hydrazones derived from aliphatic aldehydes (generated in the peptides from serine, homoserine and other amino acids containing primary -OH groups) have a serum half-life of 50-60 hours. Hydrazones derived from aromatic ketones (generated in the peptides from phenylserine and substituted phenylserines)) have a serum half-life of 130 hours. By choosing an antibody or its fragment with a half-life in human serum similar to that of hydrazone, maximum delivery of the antibody or its

fragment to the tumor is expected. After which, the release of the therapeutic unit could occur at a rate dependent on the chosen hydrazone's half-life in the tumor site's acidic environment. To prevent the polymeric carrier from possible premature degradation, the polymeric carrier can be constructed with only D-amino acids or a mixture of D- and L-amino acids.

Agents containing thiols, SH, react with cysteine side chains to form polymeric carriers with disulfide linkages having the following formula:
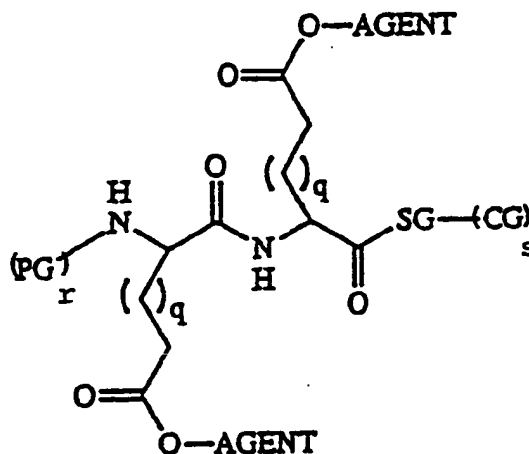
wherein   the α-amino acids in the polymeric carrier are
           from 2 to about 18 units;
           PG is an N-terminal protecting group;
           SG is a spacer group that by preventing steric
           hindrance by agents appended from the C-
           terminal end of the carrier promotes efficient
           attachment of the polymeric carrier to a
           targeting molecule;
           CG is a conjugation group useful for the
           attachment of the polymeric carrier to a
           targeting molecule;

           AGENT is from 2 to about 18 units of a
           diagnostic or therapeutic agent, or a chelating
           agent capable of binding diagnostic or
           therapeutic radionuclides in the polymeric
           carrier;
           R is H or CH_3;
           R' is H or CH_3; and
           q is 1 or 2;
           r is 0 or 1; and
           s is 0 or 1.

The release rate for agents linked through disulfide bonds
to the polymeric carrier can be decreased by replacement
of hydrogen with α-alkyl groups (R,R'=CH_3).

Agents containing hydr xyl groups react with aspartic and
glutamic acid sid chains to form p lymeric carriers with
ester linkages having the following formula:

wherein   the α-amino acids in the polymeric carrier 2 to
           about 18 units;

           PG is an N-terminal protecting group;

           SG is a spacer group that by preventing steric
           hindrance by agents appended from the C-
           terminal end of the carrier promotes efficient
           attachment of the polymeric carrier to a
           targeting molecule;

           CG is a conjugation group useful for attachment
           of the polymeric carrier to a targeting
           molecule;

           AGENT is from 2 to about 18 units of a
           diagnostic or therapeutic agent, or a chelating
           agent capable of binding diagnostic the or
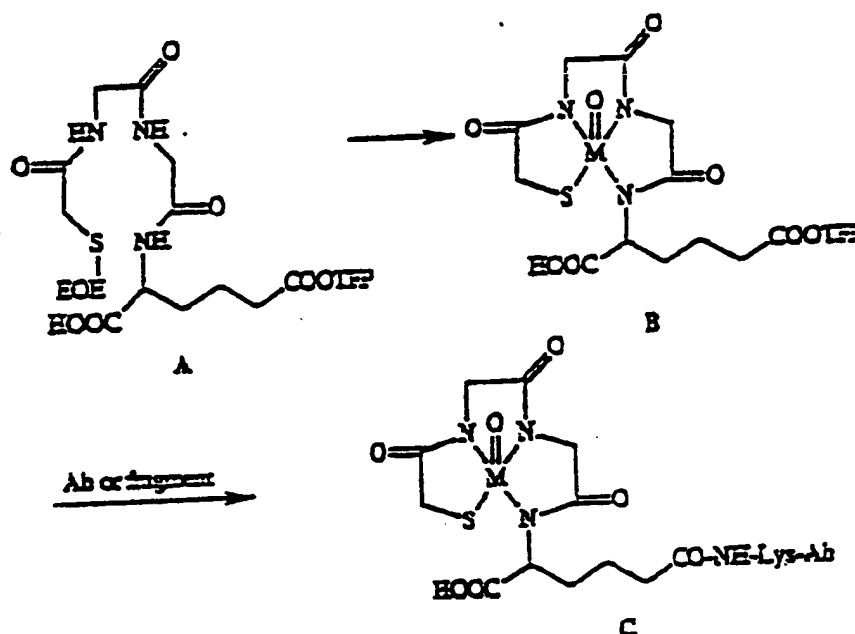   therapeutic radionuclides in the polymeric
           carrier;
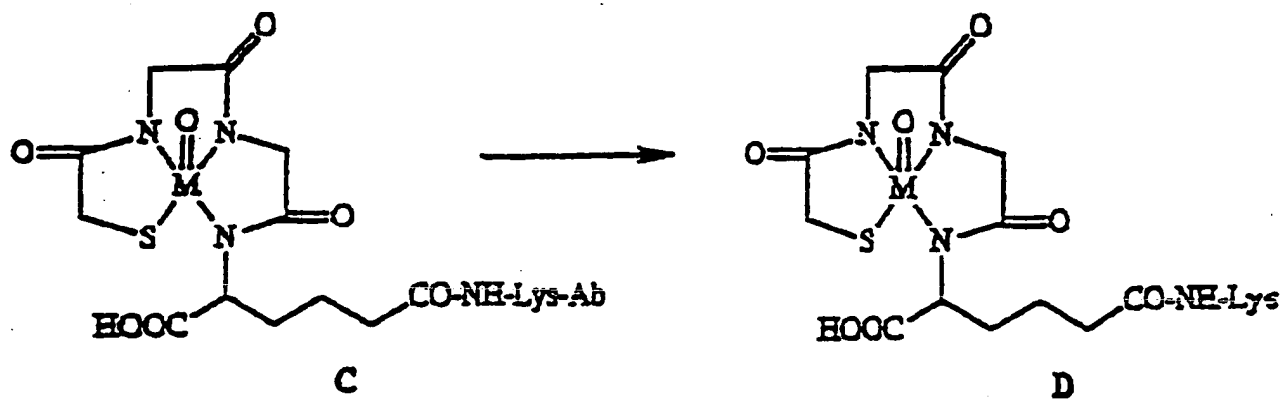
q is 0 or 1;

r is 0 or 1; and

s is 0 or 1.

In general, attachment of radionuclide metals (e.g., M = ^{99m}Tc, ^{186}Re or ^{188}Re) to monoclonal antibodies using bifunctional chelating agent A has been carried out by the following procedure. Formation of M-chelate containing active ester B followed by attachment to monoclonal antibodies to give C, according to the following reaction scheme:

EOE refers to an ethoxy ethyl protecting group. COOTFP refers to 2,3,5,6-tetrafluorophenyl ester. The above chelate is an N$_3$S derivative, and N$_2$S$_2$ derivatives follow a similar procedure. Between the chelate and the antibody

is a carbon chain attached to the α-amino group of th
antibody. After metabolism takes place in various organs,
the major metabolite D is retained in the gut and kidney
and is not excreted, according to the following:
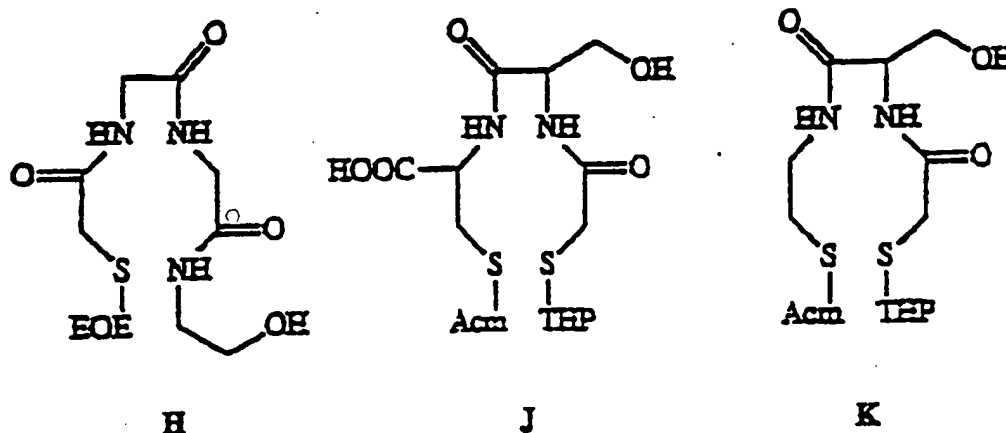
This retention interferes with imaging in th lower abdominal area and gives a high dos to th kidney during therapy. It was found that the presence of a cleavable linker between the chelate and the antibody (compound F, prepared from E) is metabolized and results in the formation of G, which has no retention in the gut and low retention in the kidney, according to the following reaction scheme:

The observation that the cleavable linker is metabolized in the above reaction scheme leads to attaching the hydroxy groups of the following compounds:

H   J   K

to the aspartic and glutamic acid side chains to form ester linkages in the polymeric carriers.

The resulting polymeric carrier can carry more than one radionuclide metal/per attachment to the antibody or fragment and offers the advantage of a metabolite that can be removed via the renal system instead of being retained in the gut.

Compound H belongs to N_3S type chelates and J and K belong to the N_2S_2 chelate system. The groups THP (tetrahydropyranyl) and Acm (acetamidomethyl) are used as sulfur protecting groups.

The targeting molecule is any molecule that will serve to deliver the polymeric carrier with attached diagnostic/therapeutic or chelating agents to a desired target site (e.g., target cells) _in vitro_ or _in vivo_. Examples of targeting molecules include, but are not limited to, steroids, cholesterol, lymphokines, and those drugs and proteins that bind to a desired target site.

The targeting molecule may be a targeting protein, which is capable of binding to a desired target site. The term "protein" as used herein includes proteins, polypeptides, and fragments thereof. The targeting protein may bind to a receptor, substrate, antigenic determinant, or other binding site on a target cell or other target site. The targeting protein serves to deliver the agent attached thereto by polymeric carrier to a desired target site _in vivo_. Examples of targeting proteins include, but are not limited to, antibodies and antibody fragments, hormones, fibrinolytic enzymes, and biologic response modifiers. In addition, other molecules that localize in a desired target site _in vivo_ although not strictly proteins, are included within the definition of the term "targeting proteins" as used herein. For example, certain carbohydrates or glycoproteins may be used in the present invention. The proteins may be modified, e.g., to produce variants and fragments thereof, as long as the desired biological property (i.e., the ability to bind to the target site) is retained. The proteins may be modified by using various genetic engineering or protein engineering techniques.

Among the preferred targeting proteins are antibodies, most preferably monoclonal antibodies. A number of

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monoclonal antibodies that bind to a specific typ of cell
have been developed including monoclonal antibodies
specific for tumor-associated antigens in humans. Among
the many such monoclonal antibodies that may be used are

5   anti-TAC, or other interleukin-2 receptor antibodies;
9.2.27 and NR-ML-05, reactive with the 250 kilodalton
human melanoma-associated proteoglycan; and NR-LU-10,
reactive with a pancarcinoma glycoprotein. The antibody
employed in the present invention may be an intact (whole)

10   molecule, a fragment thereof, or a functional equivalent
thereof.   Examples of antibody fragments are F(ab')$_2$,
-Fab', Fab, and F$_v$ fragments, which may be produced by
conventional methods or by genetic or protein engineering.

15   Proteins contain a variety of functional groups; e.g.,
carboxylic acid (COOH) or free amine (-NH$_2$) groups, which
are available for reaction with a suitable protein
conjugation group on a polymeric carrier to bind the
polymeric carrier to the targeting protein. For example,

20   an active ester on the polymeric carrier reacts with
epsilon amine groups on lysine residues of proteins to
form amide bonds. Alternatively, a targeting molecule
and/or a polymeric carrier may be derivatized to expose or
attach additional reactive functional groups. The

25   derivatization may involve attachment of any of a number
of linker molecules such as those available from Pierce
Chemical Company, Rockford, Illinois. (See the Pierce
1986-87 General Catalog, pages 313-54.) Alternatively,
the derivatization may involve chemical treatment of the

30   protein (which may be an antibody). Procedures for
generation of free sulfhydryl groups on antibodies or
antibody fragments are also known. (See U.S Patent No.

4,659,839). Maleimide conjugation groups on polymeric carriers are reactive with the sulfhydryl (thiol) groups.

Alternatively, when the targeting molecule is a carbohydrate or glycoprotein, derivatization may involve chemical treatment of the carbohydrate; e.g., glycol cleavage of the sugar moiety of a glycoprotein antibody with periodate to generate free aldehyde groups. The free aldehyde groups on the antibody may be reacted with free amine or hydrazine conjugation groups on polymeric carriers.

In the present invention, therapeutic agents (e.g., a drug, therapeutic radionuclide or toxin) are attached to the chemically defined polymeric carrier. Preferably, multiple therapeutic agents (which may be the same or different) are attached to the polymeric carrier. Exemplary therapeutic agents include toxins and drugs. Within the present invention, preferred toxins include holotoxins, such as abrin, ricin, modecin, Pseudomonas exotoxin A; Diphtheria toxin, pertussis toxin and Shiga toxin; and A chain or "A chain-like" molecules, such as ricin A, abrin A chain, modeccin A chain, the enzymatic portion of pertussis toxin, the enzymatic portion of Shiga toxin, gelonin, pokeweed antiviral protein, saporin, barley toxin, and snake venom peptides.

Exemplary drugs include daunomycin, adriamycin, vinblastine, doxorubicin, bleomycin, methotrexate, 5-fluorouracil, 6-thioguanine, cytarabine, cyclophosphamide, and similar conventional chemotherapeutics (for example, see Cancer: Principles and Practices f Oncology, 2d ed., V.T. DeVita, Jr., S.
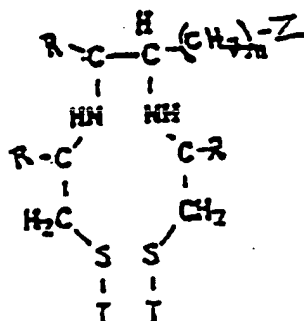
- 22 -

Hellman, S.A. Rosenberg, J.B. Lippincott Co.,
Philadelphia, PA, 1985, Chapter 14). Yet other preferred
drugs that can be used with the present invention belong
to the tricothecene family, with Roridin A particularly
5   preferred. Experimental drugs may also be suitable for
used within the present invention (see, e.g., NCI
Investigational Drugs Pharmaceutical Data 1987, NIH
Publication No. 882141, Revised November 1987).

10   In the present invention, radiolabeled molecules are
attached to the chemically defined polymeric carrier.
Preferably, a multiple number of radiolabeled molecules
(which may be the same or different) are attached to the
polymeric carrier.
15

Radionuclide metal chelates are one type of radiolabeled
molecule that may be employed. Many chelating compounds of
various structures, as well as methods for the synthesis
and radiolabeling thereof to produce radionuclide metal
20   chelates, are known. Chelating compounds comprising
various combinations of sulfur, nitrogen, oxygen, and
phosphorous donor atoms may be used, for example. The
chelating compound may, for example, comprise a total of
from four to six donor atoms selected from nitrogen and
25   sulfur atoms. During the radiolabeling procedure, bonds
form between the donor atoms and the radionuclide metal,
thereby producing a radionuclide metal chelate. Chelating
compound(s) may be incorporated into the polymeric carrier
during the synthesis procedure. Alternatively, the.
30   chelating compound(s) may be synthesized separately and
subsequently attached to the polymeric carrier.

One type of chelating compound that may be employed comprises two nitrogen and two sulfur donor atoms and thus may be designated an "N_2S_2" chelating compound. Suitable N_2S_2 chelating compounds are described in U.S. Patent number 4,897,255, entitled "Metal Radionuclide Labeled Proteins for Diagnosis and Therapy", which is hereby incorporated by reference in its entirety. One example of an N_2S_2 chelating compound is as follows:

$$
\begin{array}{c}
\overset{\displaystyle R}{\underset{\displaystyle |}{C}}-\overset{\displaystyle H}{\underset{\displaystyle |}{C}}-(CH_2)_n-Z \\
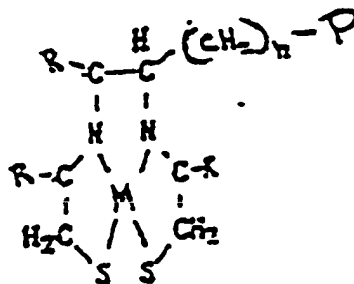\end{array}
$$

wherein n is from 1 to about 4 (preferably 2); each R independently is selected from =O and H_2; T represents a sulfur protecting group; and Z represents an active ester or other reactive functional group (which in the present invention may be useful for incorporating the chelating compound into the polymeric carrier).

Any suitable conventional sulfur protecting group(s) may be attached to the sulfur donor atoms of the compounds of the present invention. The protecting groups should be removable, either prior to or during the radiolabeling reaction. Among the preferred sulfur protecting groups are Acm and hemithioacetal protecting groups (EOE, THP), which

ar displaceable from the chelating compound during the radiolabeling reaction.

The N_2S_2 chelating compound advantageously is radiolabeled after attachment to the polymeric carrier to produce a radionuclide metal chelate of the formula:

$$R-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle N}{|}}{C}}-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle N}{|}}{C}}-(CH_2)_n-P$$

wherein P represents the polymeric carrier, M represents a radionuclide metal or oxide thereof, and the other symbols are as described above.

Radionuclide metals include, but are not limited to, the diagnostically effective radionuclide ^{99m}Tc, and the therapeutically effective radionuclides ^{188}Re, ^{186}Re, ^{67}Cu, ^{64}Cu, ^{212}Pb, ^{212}Bi, and ^{109}Pd. ^{186}Re and ^{188}Re are radionuclide metals for use in the present invention.
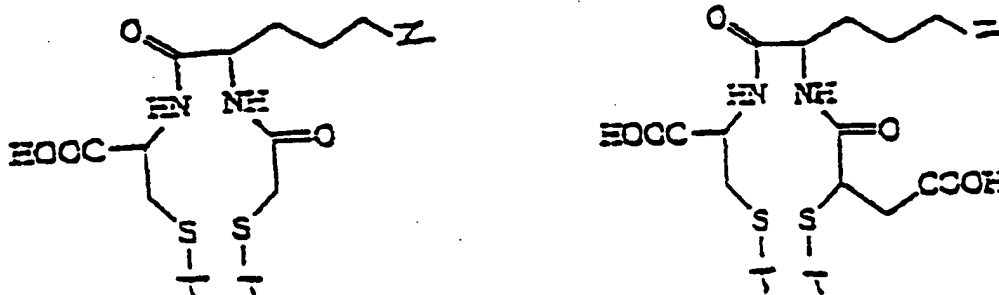
Methods for preparing these isotopes are known. Molybdenum/technetium generators for producing ^{99m}Tc are commercially available. Procedures for producing ^{186}Re include the procedures described by Deutsch et al., (Nucl.
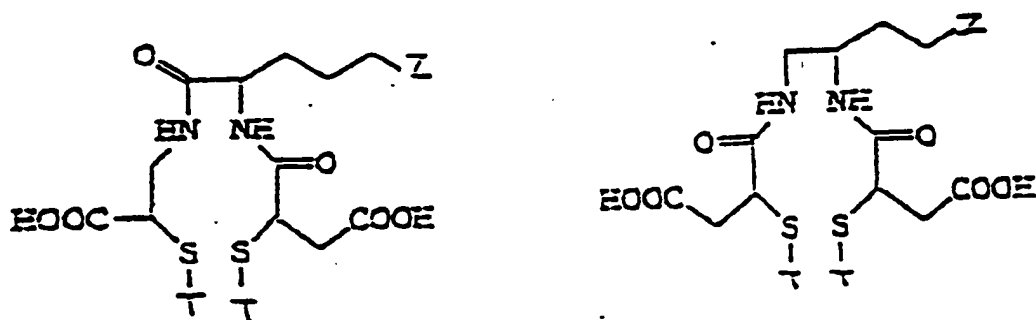
Med. Biol., Vol. _13_:_4_:465-477, 1986) and Vanderheyden et
al., (_Inorganic Chemistry_, Vol. _24_:1666-1673, 1985), and
^{212}Bi methods for production of ^{188}Re have been described by
Blachot et al. (_Intl. J. of Applied Radiation and
Isotopes_, Vol. _20_:467-470, 1969) and by Klofutar et al.
(_J. of Radioanalytical Chem._, Vol. 5:3-10, 1970).
Production of ^{109}Pd is described in Fawwaz et al., _J. Nucl.
Med._ (1984), _25_:796. Production of ^{212}Pb and ^{212}Bi is
described in Gansow et al., _Amer. Chem. Soc. Symp. Ser._
(1984), _241_:215-217, and _Kozah et al., Proc. Nat'l. Acad.
Sci._ USA (January 1986) _83_:474-478.

The radiolabeling reaction (for this N_2S_2 compound and the
other chelating compounds described below) is conducted
using conventional procedures.

Additional N_2S_2 chelating compounds comprising carboxylic
acid substituent(s) for improved biodistribution
properties are described in copending U.S. Patent
Application Serial number 07/367,502, entitled
"Radionuclide Metal Chelates for the Radiolabeling
of Proteins", which is hereby incorporated by
reference.
Examples of such chelating compounds are as follows:

wherein the symbols T and Z are as described above for the
other N_2S_2 chelating compounds.

Another type of chelating compound that may be employed
comprises one sulfur and three nitrogen donor atoms and
thus may be designated an "N_3S" chelating compound
Suitable N_3S chelating compounds include those described
in European patent application publication number 284,071
and copending U.S. Patent Application Serial number

07/172,004, both entitled "Metal-Radionuclide-Labeled Proteins and Glycoproteins for Diagnosis and Therapy", which are hereby incorporated by reference in their entirety. Ex..ples of N_3S chelating compounds include but are not limited to the following seven compounds, wherein "T" represents a sulfur protecting group and "COOTFP" represents a 2,3,5,6-tetrafluorophenyl ester group:

	R	R'	R"
Compound 1	H	H	$CH_2-CH_2-COOTFP$
Compound 2	CH_2-COOH	H	$CH_2-CH_2-COOTFP$
Compound 3	CH_2-COOH	CH_2-COOH	$CH_2-CH_2-COOTFP$
Compound 4	CH_2-CH_2-COOH	CH_2-COOH	$CH_2-CH_2-COOTFP$
Compound 5	H	$CH_2-CH_2-COOTFP$	COOH
Compound 6	$CH_2-CH_2-CH_2-COOTFP$	H	COOH
Compound 7	H	H	$CH_2-CH_2-COOTFP$

The COOTFP active ester may be replaced by other chemically reactive functional groups.

Other chelating compounds may have different combinations of donor atoms. Such compounds include among others, the N_2S_4, N_2S_3, and N_3S_3 chelating compounds described in copending U.S. Patent Application Serial no. 07/201,134, entitled "Metal Radionuclide Chelating Compounds for Improved Chelation Kinetics", which is hereby incorporated by reference in its entirety. In addition, the N_2S_2 and N_3S

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compounds presented above may c mprise varying numbers of
substituents such as carboxylic acid groups and from 0 to
3 oxygen atoms (=O) attached to carbon atoms of the
chelate core.

5

In the present invention, the chelating compounds
comprise, or are attached to, cleavable linkers. A number
of linkers that are cleavable under defined conditions
(e.g., at acidic pH, under reducing conditions, or in the
10   presence of an enzyme such as a protease) are known. The
chelates therefore may be released from the polymeric
carrier under the desired conditions.

Suitable chelating compounds comprising a cleavable link
15   age include but are not limited to those described in
copending U.S. Patent Application Serial no. 07/457,480,
entitled "Radiolabeled Proteins for Diagnostic and
Therapeutic Use", which is hereby incorporated by
reference in its entirety.   The U.S.S.N. 07/457,480
20   application discloses N_2S_2 and N_3S chelating compounds
comprising a linker of defined structure that terminates
in a chemically reactive functional group. The linkage is
cleavable at an ester group positioned in a particular
orientation therein. Examples of such chelating compounds
25   include, but are not limited to, the following:

wherein T represents a sulfur protecting group and Z represents a chemically reactive group (e.g., an active ester) which may be used to incorporate the chelating compound into the polymeric carrier in accordance with the present invention.

Other examples of radiolabeled molecules that may be attached to the polymeric carrier in accordance with the present invention include radiohalogenated molecules. Examples of molecules that bind radiohalogens at the meta or para position on a phenyl ring are described in U.S. Patent No. 4,855,153, entitled "Radiohalogenated

Proteins", which is hereby incorporated by reference in its entirety. These compounds may be represented by the following formula:

$$*X - Ar - R$$

5 wherein

*X is a radioisotope of iodine, bromine, fluorine, or astatine;

Ar is an aromatic or heteroaromatic ring; and

R is a chemical bond or a substituent containing 1

10 to 12 straight-chain carbon atoms that does not activate Ar toward electrophilic substitution on the order produced by hydroxy or amino substitution of the ring. The bond or substituent has attached thereto a chemically reactive functional group

15 useful in the present invention for incorporation of the compound (or a non-radiolabeled precursor thereof) onto the polymeric carrier. *I-paraiodophenyl compounds (in which *I represents a radioisotope of iodine) may be

20 prepared using procedures that generally involve substituting the organometallic group $Sn(n-Bu)_3$ or $SnMe_3$ on a haloaromatic compound. A radioisotope of a halogen then is substituted for the organometallic group by halodemetalization. Examples of

25 radiohalogenated molecules that may be prepared using such a procedure are represented by the following formulas:

$$*X - \!\!\!\left\langle\!\!\bigcirc\!\!\right\rangle\!\!\! - (CH_2)_n - Z$$

$$*X - \!\!\!\left\langle\!\!\bigcirc\!\!\right\rangle\!\!\! - \overset{\overset{\displaystyle O}{\displaystyle \|}}{C}NH(CH_2)_n - Z$$

wherein n repres nts an integer from 0 to 3, Z represents a reactive functional group, and *X represents a radioisotope of a halogen.

Additional radiohalogenated molecules that may be used in the present invention are described in U.S. Patent No. 4,876,081, which is hereby incorporated by reference in its entirety. The radiohalogenated molecules comprise a vinyl group.

The radiolabeled polymeric carrier targeting molecules of the present invention have use in diagnostic and therapeutic procedures, both for _in vitro_ assays and for _in vivo_ medical procedures. The radiolabeled polymeric carrier molecules may be administered intravenously, intraperitoneally, intralymphatically, locally, or by other suitable means, depending on such factors as the type of target site. The amount to be administered will vary according to such factors as the type of radionuclide (e.g., whether it is a diagnostic or therapeutic radionuclide), the route of administration, the type of target site(s), the affinity of the targeting molecule for the target site of interest, and any cross-reactivity of the targeting molecule with normal tissues.

Appropriate dosages may be established by conventional procedures and a physician skilled in the field to which this invention pertains will be able to determine a suitable dosage for a patient. A diagnostically effective dose is generally from about 5 to about 35 mCi and typically from about 10 to about 30 mCi per 70 kg body weight. A therapeutically effective dose is generally from about 20 mCi to about 300 mCi. For diagnosis,

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c nventional non-invasive procedures (e.g., gamma cameras) are used to detect the biodistribution of the diagnostic radionuclide, thereby determining the presence or absence of the target sites of interest (e.g., tumors).

To render the ester in the polymeric carrier molecules of the present invention more susceptible to cleavage in the kidneys, an agent that raises urine pH may also be administered to the patient. Such agents include, for example, a salt of ascorbate (e.g., sodium ascorbate) or a bicarbonate salt (e.g., sodium bicarbonate), which may be administered intravenously. Raising the urine pH to a basic level promotes cleavage of the ester in conjugates or catabolites thereof localized in the kidneys. Clearance of the released radionuclide metal chelates from the body is thereby enhanced. Administration of such agents to promote cleavage of ester linkers _in vivo_ is described in U.S. Patent Application Serial No. 07/251,900, which is hereby incorporated by reference.

The comparatively low intestinal localization of the therapeutic radiolabeled polymeric carrier antibodies of the present invention or catabolites thereof permits increased dosages, since intestinal tissues are exposed to less radiation. The clarity and accuracy of diagnostic images also is improved by the reduced localization of radiolabeled polymeric carrier antibodies or catabolites thereof in normal tissues.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are

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provided for purposes of illustrati n only, and are not
intended to limit the scope of the invention.

EXAMPLE 1

5   Three peptide carriers containing 6, 12, and 18 α-amino
acids with different side chain hydroxyl groups are
synthesized to show the use of hydrazones in covalently
linking to agents. The requisite peptides are synthesized
10   using the solid phase methodology of Merrifield (G. Barany
and R.B; Merrifield, "The Peptides. Analysis, Synthesis
and Biology" E. Gross and J. Meinhofer, Editors, Academic
Press, New York, pages 1-284 (1980).

15   This example first involves the synthesis of peptide 1,
N-Acetyl-L-seryl-L-aspartyl(β-Otce)-L-seryl-L-threonyl-
L-aspartyl-(β-Otce)-L-threronyl-γ-aminobutyric acid. This
is followed by the oxidation of the hydroxyl amino acid
side chain groups to carbonyl groups. These carbonyl
20   groups are then condensed with the hydrazide groups on the
agents. Next, there is formation of the active ester on
the C-terminal of the peptide. This enables the peptide
or polymeric carrier with attached agents to conjugate
with the antibody. The general procedure for the
25   synthesis of a polymeric carrier with attached agents and
the conjugation of the polymeric carrier to an antibody is
illustrated in Figure 1. Figure 2 illustrates a specific
polymeric carrier synthesis and a specific conjugation
procedure.

30   The above compounds are synthesized as C-terminal
carboxylates using PAM resin attached to the first C-
terminal amino acid (J.M. Stewart and J. Young, "Solid

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phase peptide synthesis", Pierce Chemical Company,
Rockford, Illinois, 1984)) on an Applied Biosystems 430 A
synthesizer using its specific protocols with N-
methylpyrrolidone as a coupling solvent (User's manual.
Model 430A synthesizer. Applied Biosystems, Inc., Foster
City, CA)).

The preferred protecting groups are Ser (O-benzyl), Thr
(O-benzyl), Glu(O-t-butyl), Glu(O-benzyl), Asp(O-t-butyl)
and Tyr(Br-Cbz) (G. Barany and R.B. Merrifield, "The
Peptides. Analysis, Synthesis and Biology" E. Gross and
J. Meinhofer, Editors, Academic Press, New York, pages
1-284 (1980)). The other preferred protecting group for
glutamic and aspartic acids (and other carboxyl bearing
side chains) is the trichloroethyl ester
trichloroethoxycarbonyl for tyrosine (Tce). The presence
of this protecting group on the carboxyl of Asp and Glu
residues offer protection through the sequence of
derivatization of the side chain, attachment of the agents
and the final activation of the terminal carboxyl group
for conjugation to the targeting molecule. After the
activation, the trichloroethyl group(s) can be removed by
the using Zn-HOAc or Zn-THF-phosphate buffer (R.B.
Woodward, K. Heusler, J. Gosteli, P. Naegeli, W. Oppolzer,
R. Ramage, S. Ranganathan and H. Vorbruggen, J. Amer.
Chem. Soc., 88, 852 (1989) and M.F. Sommelhack and G. E.
Heinsohn, J. Amer. Chem Soc., 94, 5139 (1972)). After
first deblocking by trifluoroacetic acid, the N-terminal
residue is acetylated using acetic anhydride and finally
cleaved from this resin using HF (See Figure 1).

The cleavage of peptides from the resin are accomplished
using the low-high HF cleavage procedure of Tam and

Merrifield (J.P. Tam, W.F. Heath and R.B. M rrifield, "SN$_2$ deprotection of synthetic peptides with low concentration of HF in dimethyl sulfide: evidence and application in peptide synthesis." J. Amer. Chem. Soc., 105, 6442 (1983)) (method A) or in 10:1:1:2 (by volume) of HF:anisole:dimethylsulfide:p-thiocresol for 1 hour at 5° to 0°C. After cleavage, the organic scavengers are extracted from the resin 3 times with ether and the peptides extracted twice with 5 mL volume of 20-40% HOAc/H$_2$O. After lyophilization, the peptides are purified on a semi-preparative Vydec LC4 reversed phase column using a gradient of 100% H$_2$O-0.1% TFA to 40% H$_2$O-0.1% TFA+60% CH$_3$CN-0.1% TFA. They are analyzed for correct amino acid composition and molecular weight by FAB mass spectrometry (T.D. Lee, "Methods of Protein Micro-characterization" J.E. Shively, editor. The Humana Press, Clifton, New Jersey, p. 403 (1986).

It is necessary to prepare the corresponding C-14 labeled peptides at the N-terminal residue acetyl for the purpose of determining the stoichiometry of attachment of peptides and modified peptides containing therapeutic molecules. The N-terminal residue after the first deblocking of the N-terminal Boc group is acetylated using labeled acetic anhydride. As an example, 10 mg of the peptide is N-acetylated with C-14-acetic anhydride (1 mCi, 11.3 mCi/mmol) which is added and shaken for 2.5 hours with the resin. A 5-fold molar excess of diisopropylethylamine was added and N-acetylation was continued for 30 minutes. Peptide resin sealed in 1 inch square polypropylene bags was washed several times with 4 mL/bag of methylene chloride, 5% diisopropylethylamine/ methylene chloride and

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finally with 10% cold acetic anhydride/methylene chloride to complete the acetylation. Excess labelled anhydride was washed from the resin by consecutive rinses of methylene chloride, dimethyl formamide, isopropanol, methylene chloride, methanol and the resin was dried overnight prior to deblocking by the procedures described above.
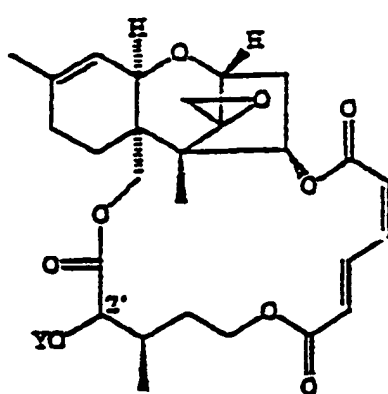
To avoid the potential proteolytic degradation of the peptide or polymeric carrier attached to the targeting molecules while in the serum, the N-terminal residue or all the residues are in the D-configuration. The change in the configuration of the peptide backbone will not alter the rate of the release of the therapeutic molecules attached to the side chain. However, this change may diminish the immunogenicity of the peptide backbone of these carriers.

The next step involves Moffatt oxidation of peptide 1 to the corresponding carbonyl compound 2. The oxidation of the peptide is carried out using DMSO, DCC, pyridine trifluoroacetate, and benzene or toluene. Moffatt oxidation is preferred for compound 2 since the procedure does not result in over oxidation of the hydroxyl compound. (For general methods see, A.F. Cook and J.G. Moffatt, J. Amer. Chem. Soc., 89, 2697 (1967) and K. E. Pfitzner and J.G. Moffatt, J. Amer. Chem Soc., 87, 5661 (1965)).
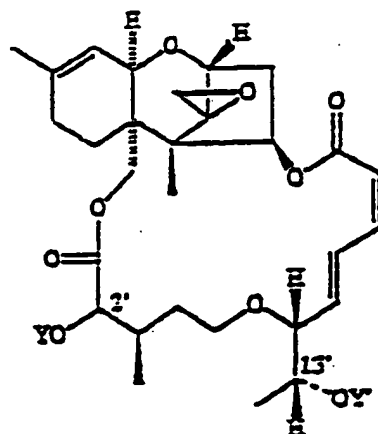
The next step is preparation (modification) of therapeutic molecules by changing their alcohol groups to hydrazides and then condensing them with modified peptides. The therapeutic molecules of interest are Verrucarin A and R ridin A which belong to the trichothecene group of

antiboiotics (B.B. Jarvis and A. Acierto in "Trichothecene Mycotoxicosis: Pathyophysiological Effects" Vol. 1. V. R. Beasley, Editor, CRC Press, Boca Raton, FL, 1989. pp. 73-105). These compounds with broad spectrum biological activities are the most potent synthesis inhibitors containing C, H and O. They exert their inhibition by interacting with the EF2 on the ribosomes (C.S. McLaughlin, M.H. Vaughen, I.M. Campbell, I.M. Wei and B.S. Hansen, "Mycotoxins in Human and Animal Health", J.V. Rodericks, Editor. Pathotox Publishers, 1977, pp 263-273). The structures of these compounds are shown below. Verrucarin A (6) and Roridin A (8) were converted to the corresponding succinyl hydrazide derivatives (7 from 6,9 and 10 from 8) according to published procedures (R. O. Kollah, "The Chemistry and Biology of Macrocyclic Trichothecenes", Ph.D. Thesis, University of Maryland, 1989; M. Zeng, "Studies in Chemical and Biological Structures of Macrocyclic Trichothecenes", Ph.D. Thesis, University of Maryland, 1989; V.M. Vrudhula, T.M. Comezoglu and A. Srinivasan, Abstract No. MEDI 50, ACS National Meeting, Boston, MA, April 1990).

A similar strategy can be used to convert other molecules of therapeutic intrest containing alcohol function to hydrazide (after incorporating a succinyl moiety) for attachment to the defined polymer 2. In a similar manner, molecules of therapeutic interest containing a carboxylic acid function can be attached to the defined polymer via the formation of a hydrazide.

Y = H Verrucarin A   6
Y = CO-(CH₂)₂-CONHNH₂   7
Verrucarin A-O-succinyl hydrazide

Y = Y' = OH Roridin A   8
Y = CO-(CH₂)₂-CONHNH₂; Y' = H   9
(Roridin A-2'-O-succinyl hydrazide)

Y = H; Y' = CO-(CH₂)₂-CONHNH₂   10
(Roridin A-13'-O-succinyl hydrazide)

Compound 6 ⟶ Compound 7

Compound 8 ⟶ Compound 9, Compound 10

In Figure 2, Macrocycle-O- CO-(CH₂)₂ = R

The next procedure is preparation of hydrazone 3 from compound 2 and Verrucarin A hydrazide 7. To a solution of the peptide (1 mmol) in isopropanol 5 mmol of Verrucarin A hydrazide (7; 10 mmol) is added and the solution is allowed to stand at room temperatures for several hours. The formation of hydrazone is followed chromatographically and is isolated either by crystallization or by C-18 column chromatography. The product is characterized by NMR and FAB mass spectrometry.

The hydrazone derived from peptide 4 and Roridin A derivatives 9 and 10 are prepared in a similar manner.

The n xt synthesis is th preparation of active ester **4** of peptide **3** (See Figure 3). To a solution of the peptide **3** in DMF, from the above reaction, 3 equivalents of 2,3,5,6-tetrafluorophenol and 3 equivalents of DCC are added and the solution is stirred at room temperature for 10-12 hours. The precipitated dicyclohexylurea is removed by filtration and the residue is chromatographed to isolate the product.

The product is dissolved in a phosphate buffer containing 10% tetrahydrofuran and the trichloroethyl groups are removed according to the procedure of M.F. Sommelhack and G.E. Heinsohn (J. Amer. Chem. Soc., $\underline{94}$ 5139 (1972)) to yield the peptide or polymeric carrier **4**, containing the therapeutic molecules and an active ester for attachment to the targeting molecule.

The last step is the conjugation of the active ester **4** to the NR-LU-10 to give the conjugate **5**. The active ester is condensed with NR-LU-10 murine monoclonal antibody, which recognizes a pancarcinoma antigen. Other proteins or fragments may be substituted for the NR-LU-10 antibody. To a solution of the antibody at pH 9-9.5, a solution of the active ester in 250 mM bicarbonate buffer at pH 9.3 is added and gently agitated to mix and incubated at room temperature for 30 minutes to allow conjugation of the peptide carrier to the antibody. The conjugate is purified in a column containing an anion exchanger DEAE-sephadex or QAE-sephadex. All of the above reactions are shown in Figure 2.

In a similar manner, conjugates are prepared from longer chain peptides, N-Acetyl-[L-seryl-L-aspartyl(β-Otce)-L-

seryl-L-threonyl-L-aspartyl-(β-Otce)-L-threonyl]$_2$-γ-aminobutyric acid, peptide **11** and N-Acetyl-L-seryl-L-aspartyl(β-Otce)-L-seryl-L-threonyl-L-aspartyl β-Otce)L-threonyl-γ-aminobutyric acid, peptide **12**.

5

General procedure for the evaluation of stability of hydrazones follows. Prior to the evaluation of conjugates the hydrazones derived from peptides **1**, **11**, and **12** are converted to the free acids **13-15** (see Figure 3). For the

10   experiments in human serum stability, the hydrazone under investigation is incubated in fresh human serum at 37°C at a concentration of 1 mg/mL. Aliquots (100 μL) at different time points (2-150 hours) are diluted with equal volumes of acetonitrile. The suspension is centrifuged and the

15   centrifugate is analyzed by HPLC for the presence of the therapeutic drug released. In a similar manner, the compounds are tested for their stability at pH 5.6.

EXAMPLE 2

This example covers the attachment of bifunctional chelate ligands to the defined peptide or polymeric conjugation of the carrier to the antibody followed by radiolabeling .

The procedures in this example are the synthesis of N-Acetyl-L-tyrosyl (O-CO-CH$_2$-CCl$_3$)-L-Asp(β-OtBu)-Glu(γ-OtBu)Gly-Glu(γ-OtBu)-γ-Aba-PAM resin 16, the removal of protecting groups from the carboxyl groups in Asp and glutamyl residues using trifluoroacetic acid to synthesize, peptide 17, the condensation of bifunctional chelate, S-ethoxyethylmercapto-acetylglycylglycylserine - trichloroethyl ester 24 to give 25, the cleavage of this peptide from the resin to give 26, the activation of the terminal carboxylic acid to give 27, the deprotection (to 28), and the radiolabeling to give the chelate 29, followed by conjugation to the antibody to yield the conjugate 30 (see Figure 4).

The requisite peptide, N-Acetyl-L-tyrosyl (O-CO-CH$_2$-CCl$_3$) - L-Asp(β-OtBu)-Glu(γ-OtBu)-Gly-Glu(γ-OtBu)-γ-Aba-PAMresin 16, is synthesized using solid phase methodology of Merrifield (G. Barany and R. B. Merrified, "The Peptides. Analysis, Synthesis and Biology" E. Gross and J. Meinhofer, Editors, Academic Press, New York, pages 1-284 (1980)). The protecting group in each step is 9-flourenylmethoxycarbonyl (Fmoc) rather than the N-tBoc group used in the synthesis of 1. This methodology conserves the protecting group of the Glu and Asp (and other amino acid residues bearing a carboxyl side chain). Removal of the Fmoc protecting group in each successive

step is accomplished by using aqueus piperdine.
Acetylation is accomplished according to the procedure
described earlier.

5   Preparation of C-14 labeled peptides at the N-terminal
residue acetyl. It is necessary to prepare the
corresponding C-14 labeled peptides for the purpose of
determining the stoichiometry of attachment of peptides
and modified peptides containing therapeutic molecules.
10   The N-terminal residue after the first deblocking of the
N-terminal Fmoc group is acetylated using labeled acetic
anhydride. As an example, 10 mg of the peptide is N-
acetylated with C-14 acetic anhydride (1 mCi, 11.3
mCi/mmol) which is added and shaken for 2.5 hours with the
15   resin. A 5-fold molar excess of diisopropylethylamine is
added and N-acetylation was continued for 30 minutes.
Peptide resin sealed in 1 inch square polypropylene bags
was washed several times with 4 mL/bag of methylene
chloride, 5% diisopropylethylamine/methylene chloride and
20   finally with 10% cold acetic anhydride/methylene chloride
to complete the acetylation. Excess labelled anhydride is
washed from the resin by consecutive rinses of methylene
chloride, dimethyl formamide, isopropanol, methylene
chloride, methanol and the resin was dried overnight prior
25   to removal of the t-Boc protecting groups.

To avoid the potential proteolytic degradation of the
peptide carrier attached to the biological macromolecules
while in the serum, the N-terminal residue or all the
30   residues are in the D-configuration. The change in the
configuration of the peptide back bone will not alter the
rate of the release of the therapeutic molecules attached

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to the side chain. This change may also diminish the immunogenicity of the peptide backbone of these carriers.

The synthesis of N-Acetyl-L-Tyr-L-Asp-Glu-Gly-Glu-γ-Aba PAM resin 17. The peptide still attached to the resin is deblocked using trifluoroacetic acid (conversion of tbutyl esters of glu and asp residues) to -COOH according to the general procedure (G. Barany and R.B. Merrifield, "The Peptides. Analysis, Synthesis and Biology" E. Gross and J. Meinhofer, Editors, Academic Press, New York, pages 1-284 (1980)).

The synthesis of S-ethoxyethylmercaptoacetyl-glycylglycylserinetrichloroethyl ester 24 (see Figure 5) involves first the synthesis of N-t-Boc-Serine-O-benzyl-trichloroethyl ester 18. To a solution of N-t-Boc-serine-O-benzyl ether (5 mmol) in methylene chloride containing 5 mmol of triethylamine, 5 mmol of N, N-dicyclohexylcarbodiimide was added and the solution was stirred at room temperature overnight. The precipitated dicyclohexylurea was filtered and the filtrate was washed with 1% HCl and water. The organic layer was dried over anhydrous Na$_2$SO$_4$, and evaporated to give the trichloroethyl ester, which was purified over a silica gel column.

Serine trichloroethyl ester trifluoroacetate 19 is prepared in the following manner. A solution of 4 mmol of the above compound 18, in 50 mL of glacial acetic acid containing 200 mg of palladium on charcoal was hydrogenated at 60 psi in a Paar apparatus for 10-12 hours. The catalyst was removed by filtration over celite and the solvent was removed in vacuo to give N-t-Boc

serine trichloroethyl ester, an oil, which was dried
overnight and used without further purification. The oil
was stirred with 10 mL 50% trifluoroacetic acid-CH_2Cl_2 for
3 hours at room temperature to remove the Boc group. The
5   mixture was evaporated to dryness, coevaporated several
times with methylene chloride and dried to give **19**. The
compound was homogeneous by TLC and was used in the next
step without further purification.

10   S-(1-ethoxylethyl) mercaptoacetic acid **20** is prepared
according to the following. A solution of mercaptoacetic
acid (17.4 mL, 250 mmol) in 125 mL of dichloromethane
containing p-toluenesulfonic acid monohydrate (0.24 g,
1.26 mmol) was cooled to -18 to -25°C with stirring. Ethyl
15   vinyl ether (23.9 mL, 250 mmol) in 125 mL of
dichloromethane was added dropwise to the cold solution
over a period of 90 minutes. The stirring was continued
for an additional 30 minutes with the temperature
maintained in the -18 to -25°C range. Then 200 mL of pH7
20   phosphate buffer was added, and the reaction mixture was
allowed to warm with stirring for 10 to 15 minutes The
mixture was then poured into a flask containing 900 mL of
theyl acetate and 200 mL of water. Layers were separated
and the aqueous portion extracted twice with ethyl
25   acetate. The organic layers were combined, washed with
brine and dried ($MgSO_4$). Removal of the solvent left 31.4
g of S-(1-ethoxyethyl) mercaptoacetic acid **20** as a
colorless oil (77% yield): ^1H NMR ($CDCl_3$) 1.15 (t, J=7.0Hz,
3H), 1.52 (d, J=6.4Hz, 3H), 3.36 (s, 2H), 3.60 (m, 2H),
30   4.84 (q, J=6.4Hz, 1H), 11.65 (s, 1H). The material was
used without further purification.

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Succinimidyl S-(1-ethoxyethyl) mercaptoacetate **21** is prepared according to the following procedure. A solution of S-(1-ethoxyethyl) mercaptoacetic acid (5.76 g, 35.1 mmol) and N-hydroxysuccinimide (4.85 g, 42.1 mmol) was prepared in 100 mL of anhydrous THF. To this was added a solution of 1,3-dicyclohexylcarbodiimide (8.70 g, 42.1 mmol) in 65 mL of anhydrous THF. The mixture was stirred at room temperature for 2 hours or until TLC analysis indicated complete formation of the succinimidyl ester. The mixture was then filtered, and the filtrate was concentrated in vacuo to a viscous residue. The residue was dissolved in ethyl acetate, washed with water, brine, and dried (MgSO$_4$). Removal of the solvent left the crude succinimidyl ester as an oil, which was further purified by flash chromatography on silica gel, using ethyl acetate-hexanes as the column eluent, to give 5.1 g of S-(1-ethoxyethyl) mercaptoacetic acid succinimidyl ester as a colorless oil (56% yield): ^{1}H NMR (CDCl$_3$) 1.21 (t, J=7.0Hz, 3H), 1.58 (d, J=6.4Hz, 3H), 2.83 (s, 4H), 3.60 (m, 4H), 4.88 (q, J=6.4Hz, 1H).

The synthesis of **22** is as follows. Solid NaHCO$_3$ (1.09 g, 13.0 mmol) was added to a solution of glycylglycine (1.22 g, 9.3 mmol) in 10 mL of water. After gas evolution ceased, a solution of (2.66 g, 10.2 mmol) in 12 mL of CH$_3$CN was added to the reaction mixture. The mixture was stirred at room temperature for 22 h, then evaporated in vacuo. The residue was purified by flash chromatography on silica gel (85:10:5 CH$_3$CN:H$_2$O:HOAc) to yield 2.2 g (86%) of **22** as a viscous oil. ^{1}H NMR (DMSO) 8.26 (t, 1H), 8.08 (t, 1H), 4.80 (q, 1H), 3.73 (m, 4H), 3.52 (m, 2H), 3.24 (s, 2H), 1.43 (d, 3H), 1.10 (t, 3H).

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The f llowing are details on the synthesis of **23**. 1,3 Dicyclohexylcarbodiimide (0.66 g, 3.2 mmol) was added to a stirring solution of **22** (0.81 g, 2.9 mmol) and N-hydroxysuccinimide (0.37 g, 3.2 mmol) in 10 mL of CH3CN.

5   After stirring for 2 h, the mixture was filtered and the filtrate was evaporated in vacuo. The residue was purified by flash chromatography on silica gel (96:4 EtOAc:HOAc) to yield 0.80 g (73%) of **23** as a viscous oil. ¹H NMR (DMSO) 8.54 (t, 1H), 8.29 (t, 1H), 4.80 (q, 1H),

10   4.27 (d, 2H), 3.78 (d, 2H), 3.53 (m, 2H), 3.24 (s, 2H), 2.81 (s, 4H), 1.43 (d, 3H), 1.09 (t, 3H).

The synthesis of S-ethoxyethylmercaptoacetylglycyl glycylserinetrichloroethyl ester **24** is prepared in the

15   following manner (see Figure 5). Triethyl-amine (2 mmol) was added to a solution of **19** (1.7 mmol) and **23** (1.7 mmol) is 5 mL of anhydrous dimethylformamide. After stirring for 2.5 hours at room temperature the mixture was evaporated in vacuo. The resulting residue was taken up

20   in ethyl acetate (20 mL) and washed with water, saturated sodium chloride and dried over sodium sulfate, filtered and evaporated. The residue was purified over a C-18 column to give pure **24**, which was used in the condensation.

25

The condensation of **24** with the -COOH residues of Glu and Asp in **17** occurs in the following manner. Solid cleavage of peptides from the resin is accomplished using the low-high HF cleavage procedure of Tam and Merrifield (J.P.

30   Tam, W.F. Heath and R.B. Merrifield, "SN₂ deprotection of synthetic peptides with low concentration of HJ in dimethyl sulfide: evidence and application in peptide

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synthesis." J. Am r. Chem. S c., 105, 6442 (1983)) (M thod A) or in 10:1:1:2 (by volume) of HF:anisole: dimethylsulfide:p-thiocresol for 1 hour at 5'-0°C. After cleavage, the organic scavengers are extracted from the resin 3 times with ether and the peptides extracted twice with 5 mL volume of 20-40% HOAc/H_2O. After lyophilization, the peptides are purified on a semi-preparative Vydec LC4 reversed phase column using a gradient of 100% H_2O-0.1% TFA to 40% H_2O-0.1% TFA+60% CH_3CN-0.1% TFA. They are analyzed for correct amino acid composition and molecular weight by FAB mass spectrometry (Ref: T.D. Lee, "Methods of Protein Microcharacterization" J.E. Shively, editor. The Humana Press, Clifton, New Jersey, p. 403 (1986)).

The following is the preparation of the active ester 27 of 26. To a solution of the peptide 26 in DMF, from the above reaction, 3 equivalents of 2,3,5,6tetrafluorophenol and 3 equivalents of DCC are added and the solution is stirred at room temperature for 10-12 hours. The precipitated dicyclohexylurea is removed by -filtration and the residue is chromatographed to isolate the product, 27.

The product 27 is dissolved in a phosphate buffer containing 10% tetrahydrofuran and the trichloroethyl groups are removed according to the procedure of M. F. Sommelhack and G.E. Heinsohn (J. Amer. Chem Soc., 94, 5139 filtration and the residue is chromatographed to isolate the product, 27.

The product 27 is dissolved in a phosphate buffer containing 10% tetrahydrofuran and the trichloroethyl

groups are removed according to the procedure of M. F. Sommelhack and G.E. Heinsohn (J. Amer. Chem Soc., 94, 5139 (1972)) to yield the peptide carrier 28, containing the chelator capable of forming metabolically stable complexes with radionuclides and an active ester for attachment to the targeting molecule.

The following is the radiolabeling procedure with ^{186}Re. The peptide containing the chelator radiolabeled with ^{186}Re according to the following procedure. Sodium perrhenate produced from a W/Re generator is combined with citric acid (a preferred complexing agent for ^{186}Re), a reducing agent (usually SnCl$_2$). The resulting ^{186}Re -citrate exchange complex is heated with the chelating compound 28 at 75°-100°C for 10-15 minutes and then transferred to a 0°C ice bath for a few minutes to obtain the peptide 29 containing ^{186}Re-complexes on the side chain.

The above solution containing the chelate is removed from the ice bath, 2.0 mL of 250 mM sodium bicarbonate buffer (pH 9-10) is added and the vial is agitated to mix. Immediately, the antibody (whole or fragments) is added and incubated at room temperature for 10-15 minutes to complete the conjugation to the antibody. The conjugate so produced is purified using an anion exchange column (DEAE-sephadex or QAE-sephadex) prepared under aseptic conditions to yield 30.

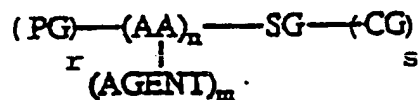In a similar approach, peptides 31 and 32 are synthesized by solid phase procedure and the antibody conjugates 37 and 38 prepared. The intermediates in the case of these oligomer syntheses are shown in Figure 6.

The inv ntion n w being fully d scribed, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made without departing from the spirit or scope of the invention.

CLAIMS

1.   A chemically defined polymeric carrier comprising a
      series of α-amino acids in any combination
      containing side chains to which agents covalently
      join through cleavable linkers either directly or
      covalently join through cleavable linkers after
      chemical modification of the side chains and
      represented by the formula:

$$(PG)_r\text{---}(AA)_n\text{---}SG\text{---}(CG)_s$$
$$\underset{(AGENT)_m}{|}$$

wherein   PG is an N-terminal protecting group;
          AA is an α-amino acid;
          SG is a spacer group that by preventing steric
          hindrance by agents appended from the C-
          terminal end of the carrier promotes efficient
          attachment of the polymeric targeting molecule;
          CG is a conjugation group useful for the
          attachment of the polymer carrier to a
          targeting molecule;
          AGENT is a diagnostic or therapeutic agent, or
          a chelating agent capable of binding diagnostic
          or therapeutic radionuclides;
          n is from 2 to about 18;
          m is from 2 to about 18;
          r is 0 or 1; and
          s is 0 or 1.

2.   The polymeric carrier f claim 1, furth r comprising at least one α-amino acid with a side chain to which an agent does not covalently join that functions as a spacer to minimize interaction between the agents covalently joined to the polymeric carrier.

3.   The polymeric carrier of claim 1, further comprising at least one α-amino acid with a charged or hydrophilic side chain to which an agent does not covalently join that provides increased solubility to the polymeric carrier.

4.   The polymeric carrier of claim 3, wherein the α-amino acid with a charged or hydrophilic side chains is selected from the group consisting of serine, threonine, lysine, arginine, histidine, cysteine, aspartic acid, glutamic acid, asparagine, glutamine, tyrosine, and tyrosine-0-SO$_3^-$.

5.   The polymeric carrier of claim 1, wherein the N-terminal protecting group, PG, is selected from the group consisting of acetyl, proprionyl, phenacyl-sulfonyl, and substituted phenacylsulfonyl.

6.   The polymeric carrier of claim 1, wherein the spacer group, SG, is selected from the group consisting of aminocaproic acid, aminopentanoic acid, γ-amino-butyric acid, β-alanine, and glycine.

7.   The polymeric carrier of claim 1, wherein the conjugation group, CG, is selected from the group consisting of active esters, isothiocyanates,

amines, hydrazines, maleimides or other Michael-
type acc ptors, thiols, and activated halides.

8.   The polymeric carrier of claim 1, wherein the
α-amino acids are all in the L configuration.

9.   The polymeric carrier of claim 1, wherein the
α-amino acids are all in the D configuration.

10.   The polymeric carrier of claim 1, wherein the
α-amino acids are any combination of L and D
configuration.

11.   The polymeric carrier of claim 1, wherein the
α-amino acid, AA, is covalently joined through a
hydrazone linkage to an agent and represented by the
formula:

wherein   PG is an N-terminal protecting group;
SG is a spacer group that by preventing
steric hindrance by agents appended from
the C-terminal end of the carrier promotes

efficient attachment of the polymeric
carrier to a targeting molecule;

CG is a conjugation group useful for the
attachment of the polymeric carrier to a
targeting molecule;

AGENT is a diagnostic or therapeutic
agent, or a chelating agent capable of
binding diagnostic or therapeutic
radionuclides;

R is H, CH_3, phenyl, or phenyl substituted
with electron-donating and/or electron-
withdrawing groups;

q is 0 or 1;

r is 0 or 1; and

s is 0 or 1.

12. The polymeric carrier of claim 1, wherein the
α-amino acid, AA, is covalently joined through a
disulfide linkage to an agent and represented by the
formula:

wherein   PG is an N-terminal protecting group;

SG is a spacer group that by preventing steric hindrance by agents appended from the C-terminal end of the carrier promotes efficient attachment of the polymeric carrier to a targeting molecule;

CG is a conjugation group useful for the attachment of the polymeric carrier to a targeting molecule;

AGENT is a diagnostic or therapeutic agent, or a chelating agent capable of binding diagnostic or therapeutic radionuclides.
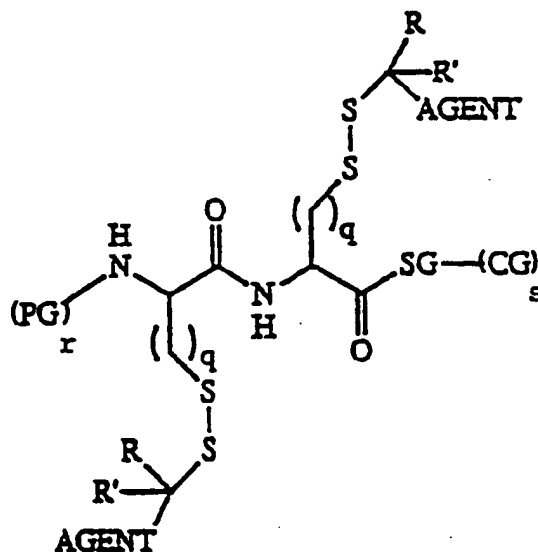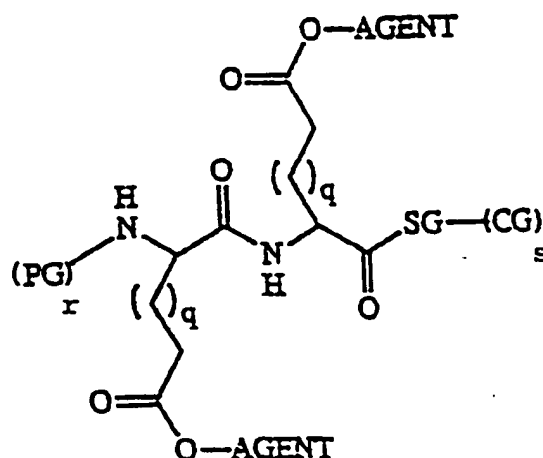
R is H or CH_3;

R' is H or CH_3;

q is 1 or 2;

r is 0 or 1; and

s is 0 or 1.

13. The polymeric carrier of claim 1, wherein the α-amino acid, AA, is covalently joined through an ester linkage to an agent and represented by the formula:

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wherein   PG is an N-t rminal protecting gr up;

SG is a spacer group that by preventing steric hindrance by agents appended from the C-terminal end of the carrier promotes efficient attachment of the polymeric carrier to a targeting molecule;

CG is a conjugation group useful for the attachment of the polymeric carrier to a targeting molecule;

AGENT is a diagnostic or therapeutic agent, or a chelating agent capable of binding diagnostic or therapeutic radionuclides;

q is 0 or 1;

r is 0 or 1; and

s is 0 or 1.

14. The polymeric carrier of claim 1, wherein the α-amino acids are covalently joined to agents through hydrazone linkages, disulfide linkages, ester linkages, and any combinations thereof.
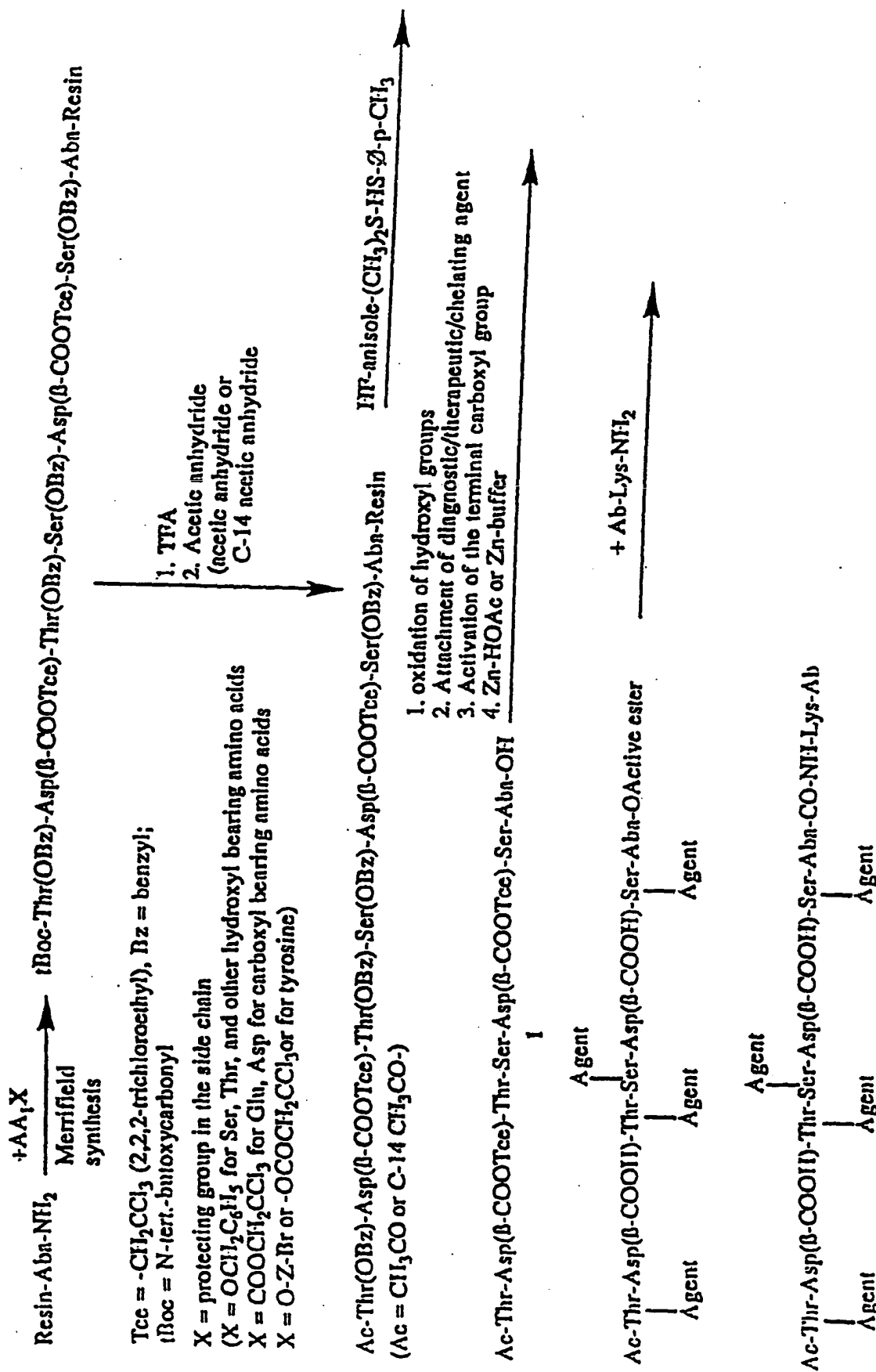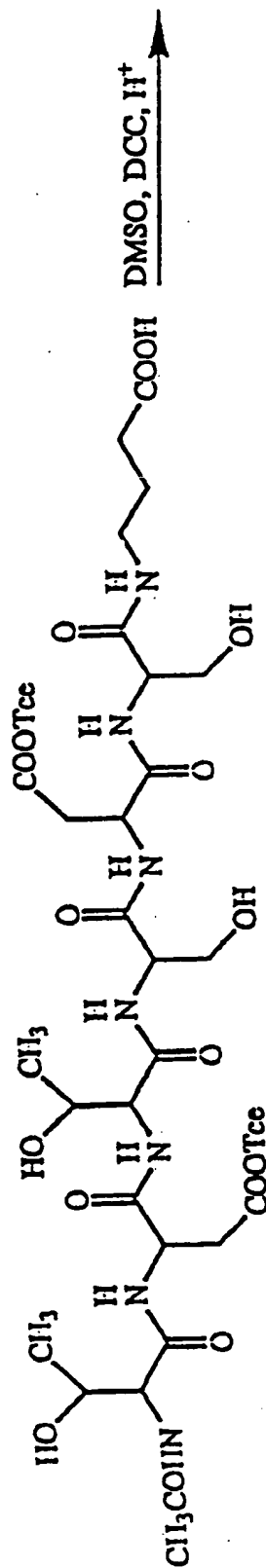
Figure 1

Resin-Abn-NH$_2$ $\xrightarrow[\text{Merrifield synthesis}]{+AA_iX}$ tBoc-Thr(OBz)-Asp(ß-COOTce)-Thr(OBz)-Ser(OBz)-Asp(ß-COOTce)-Ser(OBz)-Abn-Resin

Tce = -CH$_2$CCl$_3$ (2,2,2-trichloroethyl), Bz = benzyl;
tBoc = N-tert.-butoxycarbonyl

X = protecting group in the side chain
(X = OCH$_2$C$_6$H$_5$ for Ser, Thr, and other hydroxyl bearing amino acids
X = COOCH$_2$CCl$_3$ for Glu, Asp for carboxyl bearing amino acids
X = O-Z-Br or -OCOCH$_2$CCl$_3$or for tyrosine)

$\xrightarrow[\substack{\text{1. TFA}\\ \text{2. Acetic anhydride}\\ \text{(acetic anhydride or}\\ \text{C-14 acetic anhydride}}]{}$

Ac-Thr(OBz)-Asp(ß-COOTce)-Thr(OBz)-Ser(OBz)-Asp(ß-COOTce)-Ser(OBz)-Abn-Resin
(Ac = CH$_3$CO or C-14 CH$_3$CO-)

$\xrightarrow[\substack{\text{1. oxidation of hydroxyl groups}\\ \text{2. Attachment of diagnostic/therapeutic/chelating agent}\\ \text{3. Activation of the terminal carboxyl group}\\ \text{4. Zn-HOAc or Zn-buffer}}]{\text{HF-anisole-(CH}_3\text{)}_2\text{S-HS-ϑ-p-CH}_3}$

Ac-Thr-Asp(ß-COOTce)-Thr-Ser-Asp(ß-COOTce)-Ser-Abn-OH
|
Agent

I

$\xrightarrow{+ \text{Ab-Lys-NH}_2}$

Ac-Thr-Asp(ß-COOH)-Thr-Ser-Asp(ß-COOH)-Ser-Abn-OActive ester
| |
Agent   Agent

Ac-Thr-Asp(ß-COOH)-Thr-Ser-Asp(ß-COOH)-Ser-Abn-CO-NH-Lys-Ab
| |
Agent   Agent

Figure 2

Ac-Thr-Asp(ß-COOTce)-Thr-Ser-Asp(ß-COOTce)-Ser-Aba-OH

Figure 2(contd.)

Structure of the conjugate containing therapeutic drug attached to the defined peptide carrier attached to the antibody.

Figure 3

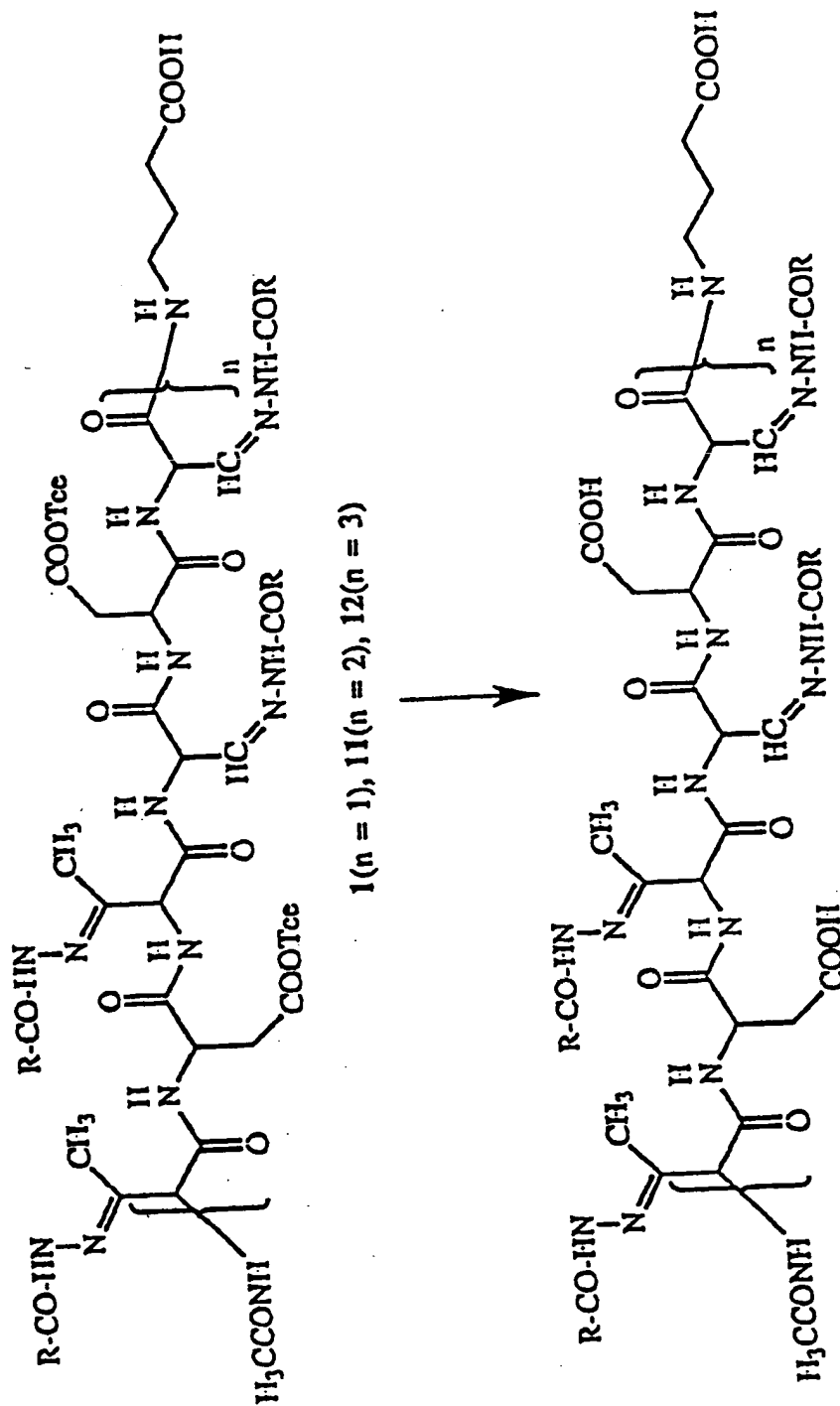
1(n = 1), 11(n = 2), 12(n = 3)

13(n = 1), 14(n = 2), 15(n = 3)

Figure 4

PAM-Abn-NH₂ $\xrightarrow{+AA_1X}$ Fmoc-tyr(O-CO-Tce)-Asp(β-OtBu)-Glu(γ-OtBu)-Gly-Glu(γ-OtBu)-γ-Abn-PAM

Merrifield synthesis

Fmoc-synthesis

AA_1X = Fmoc-protected amino acid in the synthesis
tBu = tert.butyl
Tce = 2,2,2-trichloroethyl

1. Piperidine
2. Acetic anhydride
   (C-14 acetic anhydride)

Ac-tyr(O-CO-Tce)-Asp(β-OtBu)-Glu(γ-OtBu)-Gly-Glu(γ-OtBu)-γ-Abn-PAM

16

TFA

Ac-tyr(O-CO-Tce)-Asp-Glu-Gly-Glu-γ-Abn-PAM

17

activation, LiTI 24

Ac-tyr(O-CO-Tce)-Asp-Glu-Gly-Glu-γ-Abn-PAM

25

HF-anisole

Figure 4 (contd.)

Figure 5

HOOC—CH(—O-CH₂-C₆H₅)—NH-Boc $\xrightarrow{\text{Tce-OH, DCC, NEt}_3}$ TceOOC—CH(—O-CH₂-C₆H₅)—NH-Boc

18

$\xrightarrow[\text{2. TFA}]{\text{1. [H]/Pd}}$ TceOOC—CH(—OH)—NH₂.TFA

19

HS-CH₂-COOH + CH₂=CH-O-C₂H₅ $\xrightarrow{\text{CH}_2\text{Cl}_2,\ \text{TsOH}}$ (ethoxy ring)—S-CH₂-COOH $\xrightarrow{\text{NHS, DCC}}$

20

(ethoxy ring)—S-CH₂-COOSucc. $\xrightarrow{\text{H-Gly-Gly-OH}}$ **22** $\xrightarrow{\text{NHS, DCC}}$

21

22

23 $\xrightarrow{+19}$ **24 [Li'-H]**

Figure 6

N-Acetyl-[L-Tyr-L-Asp-LGlu-Gly-LGlu]$_n$-γ-Aba-PAM resin

31 (n=2), 32 (n=3)

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 39/44, 43/00, 48/00, 49/02; C07K 15/28

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	530/345/389,402,807; 930/25,30,280; 424/1.1,9,85.91

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	US, A, 4,587,046 (GOODMAN ET AL.) 06 May 1986	1-7,10,13,14
Y	See col. 6, line 67 - column 9, line 3, and column 11, line 48 - column 14 line 30.	1-14
Y	US, A, 4,543,211 (KATO ET AL.) 24 September 1985 See column 3, line 64 - column 4, line 26; column 13, lines 25-46; and column 17-19.	1,7,10,14
Y	US, A, 4,485,093 (RUNGE) 27 November 1984 See column 2, line 60 - column 3, line 4; column 4, lines 28 - 40; and column 4, line 62 - column 5, line 66.	1,7,10
Y	US, A, 4,867,973 (GOERS ET AL.) 19 September 1989 See column 7, lines 9-15 and 35-40; column 9, lines 17-38; column 13, lines 1-10; column 22, lines 37-62; and column 23, lines 52 - column 24, line 17.	1-14
P,Y	US, A, 4,997,913 (HELLSTROM ET AL.) 05 March 1991 See column 7, line 60 - column 8, line 59; Example 5, column 15 and 16; and column 18, lines 30-38.	1-5,7-10, 12, 14

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

03 January 1992

Date of Mailing of this International Search Report

07 FEB 1992

International Searching Authority

ISA/US

Signature of Authorized Officer

John M. Covert

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	US, A, 4,732,974 (NICOLOTTI ET AL.) 22 March 1988 See entire document.	1,7,10,13,14
Y	WO, A, 87/05031 (CENTER FOR MOLECULAR MEDICINE AND IMMUNOLOGY) 27 August 1987. See page 5, paragraphs 2 and 3, and page 8, paragraphs 1-3.	

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers . because they relate to subject matter¹¹ not required to be searched by this Authority, namely:

2.☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed require-
ments to such an extent that no meaningful international search can be carried out¹³, specifically:

3.☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of
PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only
those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to
the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not
invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.